

## Biochemical Characterization of the Retinoid Isomerase System of the Eye\*

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Paul S. Bernstein‡, Wing C. Law, and Robert R. Rando§

From the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

We have previously shown that membranes from the retinal pigment epithelium can transform added all-*trans*-retinol into a mixture of 11-*cis*-retinoids, demonstrating the "missing reaction" in the visual cycle for the first time (Bernstein, P. S., Law, W. C., and Rando, R. R. (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84, 1849-1853). In this article, this isomerase activity is further characterized. Double-label experiments with [15-<sup>3</sup>H]- and [15-<sup>14</sup>C]all-*trans*-retinol as the substrate show that the tritium label is retained in the 11-*cis*-retinol and 11-*cis*-retinyl palmitate products. This requires that isomerization occur at the alcohol level of oxidation. All-*trans*-retinyl esters, such as the palmitate, acetate, butyrate, and hexanoate esters, are not directly transformed into their 11-*cis* counterparts by the membranes. The data are consistent with the presence of an all-*trans*-retinol isomerase enzyme system or enzyme complex, which produces 11-*cis*-retinol. Other isomeric retinols were tested for substrate activity. Neither 9-*cis*-retinol(al) nor 13-*cis*-retinol were processed by the isomerase.

Since the membranes containing the isomerase possess other retinol metabolizing activities, such as retinyl ester synthetase and dehydrogenase activities, further purification was attempted. Appreciable quantities of all detergents tested led to the disappearance of isomerase activity, and high salt or EDTA did not dissociate isomerase activity from the membranes. However, extensive sonication of the membranes did produce a 100,000 × *g* supernatant fraction of light membranes depleted of other all-*trans*-retinol processing activities. The isomerase activity in these membranes was saturable with all-*trans*-retinol, as required for a biologically significant process, and showed a  $V_{\max}$  of 5 pmol/h/mg of protein, a  $K_M$  of 0.8 μM, and a pH optimum of 8. The isomerase was destroyed by proteinase K, by phospholipase C, by heating, or by ethanol at concentrations greater than 1%. The addition of high energy compounds, such as MgATP, MgGTP, or palmitoyl-CoA, did not appear to stimulate isomerase activity in the 100,000 × *g* supernatant.

Rhodopsin is an integral membrane holoprotein containing 11-*cis*-retinal covalently bound to the active-site lysine by means of a protonated Schiff base (2). The absorption of light by this chromoprotein causes a *cis* to *trans* isomerization of the chromophore, with the eventual liberation of all-*trans*-retinal and opsin in a process called bleaching (3). One of the photolyzed rhodopsin intermediates, spectroscopically identified as metarhodopsin II, catalyzes the exchange of GTP for GDP in a retinal G-protein, initiating the visual transduction cascade (4). The all-*trans*-retinal produced in the retina is rapidly reduced by a rod outer segment-associated alcohol dehydrogenase and transported to the pigment epithelium, where it is esterified to long chain fatty acid esters (5). In order for vision to proceed after a strong bleach, 11-*cis*-retinal must be resynthesized and combined with opsin to form rhodopsin. A key transformation in the composite of chemical reactions comprising the visual cycle is the thermal isomerization of an all-*trans*-retinoid to its 11-*cis* congener. This "missing reaction" of the visual cycle has recently been described for the first time in an *in vitro* system (1). We have found that added all-*trans*-retinol can be converted into a mixture of 11-*cis*-retinoids by pigment epithelium/retinal membranes from the amphibian eye (1). The heat-sensitive activity was found to be located largely, if not entirely, in the pigment epithelium membranes, and it was found to use free all-*trans*-retinol as the preferred substrate (1).

Given the metabolic complexity of the membranes, it is of some interest to characterize them with respect to substrate specificity. Double-label experiments using [15-<sup>3</sup>H]- and [15-<sup>14</sup>C]all-*trans*-retinol show that <sup>3</sup>H is not lost during isomerization. Hence, all-*trans*-retinal cannot be the substrate for isomerization. Esters of all-*trans*-retinol, although hydrolyzed by the membranes, are not directly isomerized to their 11-*cis* counterparts by the membranes. With respect to the specificity of the isomerase toward the retinols, evidence is presented that only all-*trans*-retinol is processed to 11-*cis*-retinoids by the membranes.

The metabolic complexity of the membranes with respect to the metabolism of all-*trans*-retinol made it desirable to simplify the system in order to characterize the isomerase. For example, it would be impossible to quantify any kinetic characteristics of the isomerase if the majority of the added substrate were being utilized by other competing processes. Although detergents proved not to be useful in the studies described here, extensive sonication and isolation of the light membrane fraction by differential centrifugation produced material suitable for kinetic characterization. Highly sonicated membranes were still capable of carrying out the isomerization reaction with added all-*trans*-retinol, but showed minimal abilities to esterify or oxidize the added all-*trans*-retinol. Saturation kinetics were observed with added all-*trans*-retinol, and the observed  $V_{\max}$  was 5 pmol/h/mg of

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§ To whom reprint requests should be addressed: Dept. of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 250 Longwood Ave., Boston, MA 02115.

protein with a  $K_M$  of 0.8  $\mu\text{M}$ . The pH optimum was 8.0. Neither all-*trans*-retinal nor all-*trans*-retinyl palmitate were isomerized. The isomerase activity was irreversibly destroyed by proteinase K, by phospholipase C, by several detergents, and by ethanol.

## MATERIALS AND METHODS

Unless otherwise mentioned, all procedures were performed under dim red light with samples kept on ice.

**Preparation of Radioactive Retinoids**—[11,12- $^3\text{H}$ ]all-*trans*-Retinol (53 Ci/mmol), [11,12- $^3\text{H}$ ]all-*trans*-retinyl acetate (53 Ci/mmol), and [15- $^{14}\text{C}$ ]all-*trans*-retinoic acid (44 mCi/mmol) were purchased from Amersham Corp. [15- $^3\text{H}$ ]all-*trans*-Retinol (29.0 Ci/mmol) was from Du Pont-New England Nuclear. [11,12- $^3\text{H}$ ]all-*trans*-Retinal (5 Ci/mmol) and [11,12- $^3\text{H}$ ]9-*cis*-retinal (5 Ci/mmol) were prepared by standard methods from [11,12- $^3\text{H}$ ]all-*trans*-retinol (6). [11,12- $^3\text{H}$ ]all-*trans*-Retinyl palmitate (5 Ci/mmol), [11,12- $^3\text{H}$ ]all-*trans*-retinyl butyrate (5 Ci/mmol), and [11,12- $^3\text{H}$ ]all-*trans*-retinyl hexanoate (5 Ci/mmol) were prepared by standard methods using the appropriate acid chloride (Aldrich) and [11,12- $^3\text{H}$ ]all-*trans*-retinol (7). The isomeric purities were >95%. [11,12- $^3\text{H}$ ]13-*cis*-Retinol (5 Ci/mmol) was prepared by photoisomerization of [11,12- $^3\text{H}$ ]all-*trans*-retinal in methanol, followed by reduction with  $\text{NaBH}_4$  and HPLC<sup>1</sup> separation of the 13-*cis*-retinol (6). The isomeric purity was >90%.

**Preparation of Isomerase-containing Membranes**—The procedure for preparation of frog (*Rana pipiens*) retina/pigment epithelium has been described (1). The protein content of the 600  $\times g$  supernatant was 4–8 mg/ml. In some experiments, the 600  $\times g$  supernatant was washed by two successive pelletings at 50,000  $\times g$  for 20 min at 4  $^\circ\text{C}$ , followed by resuspension each time in the original volume of buffer. The protein content of this membrane preparation was 1.5–3 mg/ml.

The procedure for preparation of bovine pigment epithelium homogenate has been described (8). 50 ml of crude bovine pigment epithelium homogenate from 25 calf eyes was centrifuged at 1,500  $\times g$  for 15 min. The supernatant was then centrifuged at 150,000  $\times g$  for 75 min, and the resulting supernatant was then discarded. The pellet (15 mg of protein) was washed and resuspended in 5 ml of 50 mM sodium phosphate buffer (pH 7.2) for storage at  $-80^\circ\text{C}$ .

**Isomerase Assay and Analysis of Products**—The detailed procedure for production and analysis of  $^3\text{H}$ -retinoids was described in detail elsewhere (1). Typically, 1  $\mu\text{Ci}$  of [11,12- $^3\text{H}$ ]all-*trans*-retinol was incubated with 300  $\mu\text{l}$  of frog retina/pigment epithelium homogenate. Fifteen  $\mu\text{l}$  of 10% (w/v) solution of defatted BSA (Sigma) was added as the retinoid carrier. The incubation was carried out at room temperature for 3 h. One-third of the incubation mixture was withdrawn and extracted by *n*-hexane for the analysis of the isomeric composition of the [ $^3\text{H}$ ]retinols. The remainder of the incubation mixture was subjected to  $\text{NH}_4\text{OH}/\text{CH}_2\text{Cl}_2$  extraction (9). The extracts were used for the analysis of the isomeric composition of [ $^3\text{H}$ ]retinal oximes and [ $^3\text{H}$ ]retinyl palmitates. In some experiments, the composition of total retinoids was analyzed by gradient HPLC and time-based collection as described previously (10). Retinyl acetates were analyzed with 1% ether in hexane. However, under these HPLC conditions the 11-*cis*- and 13-*cis*-isomers coeluted. Retinyl butyrates and hexanoates were analyzed with 0.5% ether in hexane. Under these conditions, 11-*cis*-retinyl butyrate coeluted with all-*trans*-retinyl oleate, and 11-*cis*-retinyl hexanoate coeluted with all-*trans*-retinyl palmitate. Both all-*trans*-retinyl palmitate and oleate were formed by the incubation of all-*trans*-retinyl butyrate and hexanoate with frog retina/pigment epithelium homogenate. In the above cases, the actual isomeric composition of the retinoid esters was determined by first saponifying the collected substrate ester peaks with 5%  $\text{KOH}/\text{MeOH}$  at 0  $^\circ\text{C}$  for 0.5 min. The retinols were separated and their isomeric composition was determined by standard HPLC methods (1).

It should be noted that the concentration of all-*trans*-retinol in a standard assay is approximate one-tenth of the  $K_M$  of 0.8  $\mu\text{M}$  determined later in this paper. This low concentration of labeled substrate was used in all assays except for the kinetic constant determinations for two reasons. First, it was desired to retain consistency with our previously published data (1). Secondly, in a radioactive assay such

as this, operating at far below the  $K_M$  maximizes percent conversion of the labeled substrate, and this substantially improves the signal-to-noise ratio of the product on the analytical radiochromatograms.

**In Vitro Double-labeling Experiments**—A mixture of [15- $^3\text{H}$ ]- and [15- $^{14}\text{C}$ ]all-*trans*-retinol containing 0.1  $\mu\text{Ci}$  of  $^3\text{H}$  ( $^3\text{H}$ : $^{14}\text{C}$ , 5.2:1) in 100  $\mu\text{l}$  of 10% BSA (as retinoid carrier) was incubated with 4 ml of either retina/pigment epithelium 600  $\times g$  supernatant or washed 600  $\times g$  membrane preparation from four light-adapted *Rana pipiens* frogs at room temperature for 3 h. The retinoids were analyzed by the standard methods already described. The same amount of  $^3\text{H}$ - and  $^{14}\text{C}$ -labeled all-*trans*-retinol was incubated with 3 mg of protein from bovine pigment epithelium suspended in 4 ml of 50 mM phosphate buffer (pH 7.2) with 100  $\mu\text{l}$  of 10% BSA as retinoid carrier at 35  $^\circ\text{C}$  for 1 h. The retinoids were analyzed by standard methods.

**Attempted Detergent Solubilization of Retinoid Isomerase Activity**—A 600  $\times g$  frog retina/pigment epithelium supernatant was prepared as described above. 30  $\mu\text{l}$  of a concentrated detergent solution in 50 mM phosphate buffer (pH 7.2) was added to 300  $\mu\text{l}$  of 600  $\times g$  supernatant to give a detergent concentration of 0.1–1.5%. After approximately 5 min of incubation at room temperature the material was centrifuged at 13,000  $\times g$  for 10 min at 4  $^\circ\text{C}$ . The modified isomerase assay using hexane extraction and HPLC analysis was performed on 200  $\mu\text{l}$  of the supernatant as described below for the Percoll gradient experiments.

**Percoll Gradient Centrifugation of Membranes from Frog Pigment Epithelium**—The pigment epithelium from four dark-adapted frog eyes was homogenized in 2 ml of 50 mM phosphate buffer (pH 7.2) containing 0.25 M sucrose by a microultrasonic cell disrupter (Kontes) at full power for 10 s. Nuclei, pigment granules, and unbroken cells were removed by two 10-min centrifugations at 600  $\times g$ . The particulate material from the 600  $\times g$  supernatant was then sedimented by centrifugation at 100,000  $\times g$  for 1 h at 4  $^\circ\text{C}$  in a preparative ultracentrifuge. The supernatant was discarded, and the pellet was resuspended in 0.25 ml of the phosphate/sucrose buffer by brief sonication. The resuspended membrane material was transferred to a 5-ml Beckman Quick-Seal centrifuge tube.

Percoll density gradient medium (Pharmacia LKB Biotechnology Inc., density 1.28 g/ml) was made isoosmotic by addition of 1 part 2.5 M sucrose to 9 parts Percoll. The stock isoosmotic Percoll was then diluted with 4 volumes of 50 mM phosphate buffer (pH 7.2) containing 0.25 M sucrose. This 20% Percoll solution was then used to fill the 5-ml centrifuge tube containing the pigment epithelium membrane material. A balance tube with the 20% Percoll and Density Marker Beads (Pharmacia) was also prepared according to the manufacturer's instructions. These are required to indicate the various gradient densities since Percoll gradients are nonlinear. The tubes were heat-sealed and then centrifuged at 60,000  $\times g$  for 30 min at 4  $^\circ\text{C}$  in a vertical rotor. After centrifugation a single broad membrane band was visible half way down the gradient between densities of 1.037 and 1.049 mg/ml. The contents of the gradient tube were fractionated by cutting off the top of the tube and then piercing the bottom with a syringe needle. Ten 0.5-ml fractions were collected.

Isomerase assays on each fraction were performed as described above for the 600  $\times g$  supernatants with slight modifications. Each assay tube contained 200  $\mu\text{l}$  of gradient material, 10  $\mu\text{l}$  of 10% BSA (w/v), and 1  $\mu\text{Ci}$  of [11,12- $^3\text{H}$ ]all-*trans*-retinol (Amersham Corp., 60 Ci/mmol) in 1  $\mu\text{l}$  of ethanol. The  $\text{NH}_4\text{OH}/\text{CH}_2\text{Cl}_2$  extraction method required for complete retinoid extraction (1) was used. Protein assays on each fraction were performed according to the Peterson modification of the Lowry method (11). Fractions from a Percoll gradient with no membrane material were used as blanks because Percoll forms a background color in a Lowry assay (manufacturer's data). In fractions with high Percoll content, excessive turbidity necessitated brief centrifugation before putting the samples in the spectrophotometer for the protein assay.

**Preparation and Assay of a 100,000  $\times g$  Supernatant from Frog Retina/Pigment Epithelium**—The retina/pigment epithelium from eight light-adapted frog eyes were placed in four 2-ml centrifuge tubes. 0.45 ml of 50 mM phosphate buffer (pH 7.2) was added to each tube, and the material in each tube was homogenized by 10 s of sonication at full power by a microultrasonic cell disrupter (Kontes). The homogenates were then centrifuged at 600  $\times g$  for 10 min at 4  $^\circ\text{C}$ . The supernatants were divided between two 2-ml centrifuge tubes, and they were resonicated and recentrifuged as described above. The supernatants were combined in a 2-ml tube which was centrifuged at 100,000  $\times g$  for 1 h at 4  $^\circ\text{C}$ . The 100,000  $\times g$  supernatant was then dialyzed overnight against 600 ml of phosphate buffer in dialysis tubing with a molecular weight cutoff of 13,000 in an attempt to

<sup>1</sup> The abbreviations used are: HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CoA, coenzyme A; PMSF, phenylmethylsulfonyl fluoride.



remove any soluble low molecular weight cofactors. After dialysis the material could be stored at 4 °C for at least 1 week.

In a standard isomerase assay 100  $\mu$ l of 100,000  $\times$  *g* supernatant was put into a 2-ml centrifuge tube. 100  $\mu$ l of 50 mM phosphate buffer (pH 7.2) was added, along with 10  $\mu$ l of 10% BSA (w/v) and 1  $\mu$ Ci of [11,12- $^3$ H]all-*trans*-retinol (60 Ci/mmol) in 1  $\mu$ l of ethanol. Tubes were wrapped in foil and incubated at room temperature on a Clay Adams Nutator (Fisher Scientific) for 2 h. At the end of an incubation, 400  $\mu$ l of methanol and 200  $\mu$ l of water were added, followed by 600  $\mu$ l of hexane containing 1 mg/ml butylated hydroxytoluene. After brief centrifugation 120- $\mu$ l portions of the hexane extract were mixed with an isomeric retinol standard and analyzed by HPLC as described for a 600  $\times$  *g* supernatant.

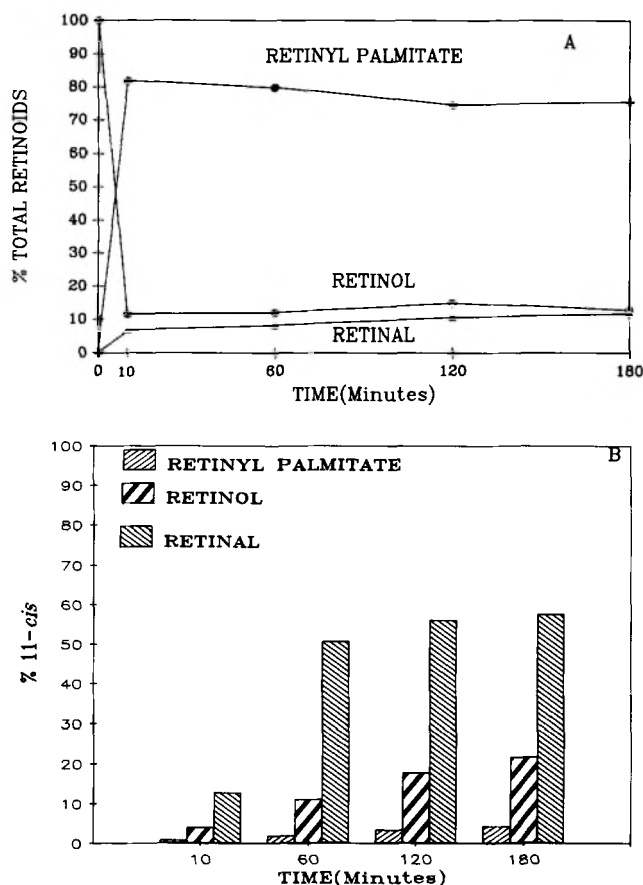
**11-*cis*-Retinoid Production in Frog Eye Homogenates from [ $^3$ H]all-*trans*-Retinal and [ $^3$ H]all-*trans*-Retinyl Palmitate**—[11,12- $^3$ H]all-*trans*-Retinal (6 Ci/mmol) and [11,12- $^3$ H]all-*trans*-retinyl palmitate (60 Ci/mmol) were prepared by standard methods (6) from [11,12- $^3$ H]all-*trans*-retinol. All products were purified by HPLC before use. 100,000  $\times$  *g* frog eye supernatants were prepared and incubated as described above, with the exception that 0.1  $\mu$ Ci of [ $^3$ H]all-*trans*-retinal was added instead of the usual 1  $\mu$ Ci used for the other retinoids because of its lower specific activity. The  $\text{NH}_2\text{OH}/\text{CH}_2\text{Cl}_2$  extraction method was used, and gradient HPLC analysis was performed as described previously (10).

## RESULTS

**Free all-*trans*-Retinaldehyde Is Not the Isomerase Substrate**—In our previous studies, we had shown that all-*trans*-retinol was processed to afford mixtures of 11-*cis*-retinol, 11-*cis*-retinal, 11-*cis*-retinyl palmitate, and larger amounts of all-*trans*-retinyl palmitate, by a membrane fraction largely associated with the amphibian pigment epithelium (1). Fig. 1A shows a time course for the processing of [ $^3$ H]all-*trans*-retinol by these membranes, and Fig. 1B shows a time course for the formation of the relevant 11-*cis* isomers. As can be seen here, the 11-*cis*-retinyl palmitate is formed most sluggishly, and the 11-*cis*-retinal most rapidly, with 11-*cis*-retinol intermediate between the two. However, in terms of absolute amount, the three populations of 11-*cis*-retinoids were generally approximately equal (1). It is also noteworthy that approximately 80% of the added substrate is rapidly converted into all-*trans*-retinyl palmitate by the membranes. Thus, as previously observed (1), these membranes contain both retinol dehydrogenase and retinol ester synthetase activities, in addition to the isomerizing activity.

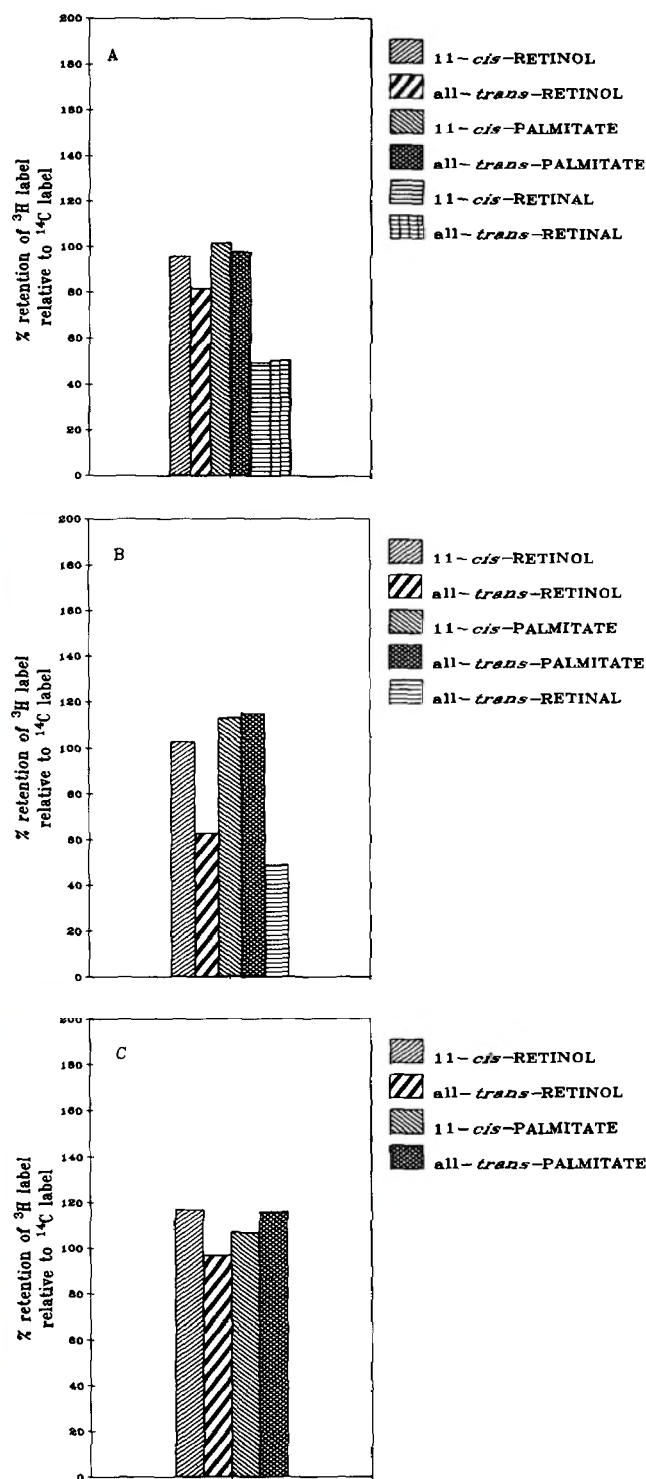
Given the metabolic complexity of the membranes with respect to the metabolism of the retinoids, the fact that added all-*trans*-retinol is converted into 11-*cis*-retinoids cannot be taken as evidence that retinol is the substrate for the isomerase. Previously, we developed a double-labeling method to reveal the oxidation state of the isomerization substrate *in vivo* (10). The method involves following the fate of the nonstereospecifically labeled  $^3\text{H}$  of [15- $^3\text{H}$ ]- and [15- $^{14}\text{C}$ ]all-*trans*-retinol during the isomerization process (10). If isomerization occurred at the alcohol level of oxidation, the initially formed 11-*cis*-retinol(palmitate) should not lose any  $^3\text{H}$ . On the other hand, if free retinaldehyde were the isomerase substrate, one-half of the  $^3\text{H}$  would be lost in the products (10). As shown in Fig. 2, A-C, with membranes from the amphibian retina/pigment epithelium and membranes from bovine pigment epithelium, the isomerization to form 11-*cis*-retinol(palmitate) occurs with complete retention of  $^3\text{H}$ . A  $^3\text{H}/^{14}\text{C}$  of even slightly greater than 1 was often observed. As expected, the retinaldehydes formed had lost one-half of their  $^3\text{H}$ . These results are only compatible with the free substrate being at the alcohol level of oxidation. These results are in complete agreement with our previous *in vivo* results on the rat (10).

**all-*trans*-Retinyl Esters Are Not Directly Isomerized to 11-*cis*-Retinyl Esters**—The question then becomes, is it the free



**FIG. 1. Formation of retinoids with time from the incubation of [11,12- $^3$ H]all-*trans*-retinol with frog retina/pigment epithelium 600  $\times$  *g* supernatant.** Four identical samples, each containing 1  $\mu$ Ci of [11,12- $^3$ H]all-*trans*-retinol, 300  $\mu$ l of frog retina/pigment epithelium 600  $\times$  *g* supernatant, and 15  $\mu$ l of 10% BSA, were assayed at the indicated times of 10, 60, 120, and 180 min for: A, the composition of retinoids with time and B, the formation of 11-*cis*-retinoids with time.

ester or the free alcohol that is the substrate for the isomerase? We had previously demonstrated that the membranes were not capable of converting added all-*trans*-retinyl palmitate to any 11-*cis*-retinoids (1). On the surface, this would appear to eliminate the ester as a possible substrate. However, one must consider the extreme hydrophobicity of retinyl palmitates and be concerned that they might not distribute to where the isomerizing activity is present (12). Because of this, less hydrophobic esters of all-*trans*-retinol were studied. When [ $^3$ H]all-*trans*-retinyl acetate was added to the membranes, rapid hydrolysis occurred, to yield results virtually identical to those obtained with added all-*trans*-retinol (Fig. 1, A and B) since there is rapid enzymatic hydrolysis of the ester. However, even after 3 h incubation with the membranes, approximately 10% of the acetate ester remained. Since the HPLC separation system used to separate acetate esters did not separate the 11-*cis*- and 13-*cis*-retinyl acetate esters, the corresponding peak was collected and hydrolyzed with 5% KOH/MeOH at 0 °C for 0.5 min. The resulting retinoids were then analyzed by HPLC. This analysis showed that significant amounts of 11-*cis*-retinyl acetate were not formed (Table I). Similar experiments were performed with the butyrate and hexanoate esters of [ $^3$ H]all-*trans*-retinol, with basically the same results, although the enzymatic hydrolysis of the esters occurred much more slowly. After 5 h incubation with the membranes, only 60% of the added butyrate ester was hydro-



**FIG. 2. Double-label experiments:** A, on frog retina/pigment epithelium  $600 \times g$  supernatant; B, on frog retina/pigment epithelium washed  $600 \times g$  particulate fraction; and C, on bovine pigment epithelium  $150,000 \times g$  particulate fraction. A, a mixture of [ $^{15}\text{H}$ ]- and [ $^{15}\text{C}$ ]all-*trans*-retinol containing  $0.1 \mu\text{Ci}$  of  $^3\text{H}$  ( $^3\text{H}$ : $^{14}\text{C}$ , 5.2:1) was incubated with 4 ml of retina/pigment epithelium  $600 \times g$  supernatant from four light-adapted *Rana pipiens* frogs, and  $100 \mu\text{l}$  of 10% BSA for 3 h at room temperature. The retinoids were analyzed by standard methods. B, the washed  $600 \times g$  particulate fraction was prepared by two successive pelletings of the  $600 \times g$  supernatant at  $50,000 \times g$  for 20 min at  $4^\circ\text{C}$ , followed by resuspension each time in the original volume of buffer. A mixture of [ $^{15}\text{H}$ ]- and [ $^{15}\text{C}$ ]all-*trans*-retinol containing  $0.1 \mu\text{Ci}$  of  $^3\text{H}$  ( $^3\text{H}$ : $^{14}\text{C}$ , 5.2:1) was incubated with 4 ml of retina/pigment epithelium washed  $600 \times g$  particulate fraction from four light-adapted *Rana pipiens*

TABLE I

Studies on the formation of 11-*cis*-retinyl isomers from the incubation of [ $^{11,12}\text{H}$ ]all-*trans*-retinyl esters with frog retina/pigment epithelium  $600 \times g$  supernatant at room temperature

The incubation mixture included 1 ml of  $600 \times g$  supernatant, 50  $\mu\text{l}$  of 10% BSA, 6  $\mu\text{Ci}$  of [ $^{11,12}\text{H}$ ]all-*trans*-retinyl acetate, or 0.6  $\mu\text{Ci}$  of [ $^{11,12}\text{H}$ ]all-*trans*-retinyl butyrate, or 0.6  $\mu\text{Ci}$  of [ $^{11,12}\text{H}$ ]all-*trans*-retinyl hexanoate; incubation time was 3 h for acetate, 4.5 h for butyrate, and 15 h for hexanoate. Under these conditions, 90% of the acetate, 60% of the butyrate, 30% of the hexanoate were hydrolyzed. The unhydrolyzed esters were extracted by hexane and the isomeric compositions were analyzed.

all- <i>trans</i> - Retinyl ester	Ester radioactivity eluting with the 11- <i>cis</i> isomer	Isomeric composition of retinol obtained by hydrolysis of the 11- <i>cis</i> -ester peak <sup>a</sup>			11- <i>cis</i> calculated from hydrolysis experiment
		11- <i>cis</i>	13- <i>cis</i>	all- <i>trans</i>	
	%	%	%	%	actual %
Acetate (without homogenate)	12 <sup>b</sup>				
Acetate (with homogenate)	19.8 <sup>b</sup>	7.4	91.8	0.8	1.5
Butyrate (without homogenate)	1.7				
Butyrate (with homogenate)	8.4 <sup>c</sup>	2.2	5.5	92.3	0.2
Hexanoate (without homogenate)	1.7				
Hexanoate (with homogenate)	9.4 <sup>d</sup>	2.2	2.4	95.6	0.2

<sup>a</sup> Since the 11-*cis*-retinyl esters coelute with other compounds formed during the incubation, the 11-*cis*-ester peaks were collected by HPLC and hydrolyzed with 5% KOH/MeOH at  $0^\circ\text{C}$  for 0.5 min. The retinol isomers formed were separated by HPLC and analyzed.

<sup>b</sup> The 11-*cis* and 13-*cis*-isomers coelute.

<sup>c</sup> The 11-*cis*-retinyl butyrate coelutes with all-*trans*-retinyl oleate.

<sup>d</sup> The 11-*cis*-retinyl hexanoate coelutes with all-*trans*-retinyl palmitate.

lyzed, and only 30% of the hexanoate ester was hydrolyzed after 15 h incubation. HPLC analysis of the esters, after hydrolysis to the corresponding retinols, indicated that again there was no direct ester-ester isomerization (Table I).

**Specificity of the Isomerase for the Retinols**—The experiments described above are consistent with the hypothesis that free all-*trans*-retinol is the substrate for the isomerase. It was of interest to further explore the substrate specificity with respect to the retinols. Addition of [ $^3\text{H}$ ]9-*cis*-retinol to the amphibian membranes did not lead to significant formation of 11-*cis*-retinoids over control, even though greater than 40% of the retinal was enzymatically processed by reduction and esterification (Table II). The situation is more complicated with [ $^3\text{H}$ ]13-*cis*-retinol, because unwashed membranes appear to convert this material to the 11-*cis*-retinoids virtually as well as all-*trans*-retinol (Table II). However, when the membranes were washed (1), to rid them of the nicotinamide adenine dinucleotide cofactors, the conversion to 11-*cis*-retinoids was sharply reduced (Table II). The same treatment does not affect isomerization when all-*trans*-retinol is the substrate (Table II). We interpret this to mean that most, if

frogs at room temperature for 3 h. The retinoids were analyzed by standard methods. No appreciable radioactivity associated with 11-*cis*-retinol was found. C, the same amount of  $^3\text{H}$ - and  $^{14}\text{C}$ -labeled all-*trans*-retinol in  $100 \mu\text{l}$  of 10% BSA (as retinol carrier) was incubated with 3 mg of protein from bovine pigment epithelium suspended in 4 ml of 50 mM phosphate buffer (pH 7.2) at  $35^\circ\text{C}$  for 1 h. The retinoids were analyzed by standard methods. This homogenate preparation is essentially free of redox activity since appreciable quantities of 11-*cis*- and all-*trans*-retinal were not formed. All four retinoids isomers analyzed completely retained their  $^3\text{H}$  label.

TABLE II

*In vitro* formation of 11-*cis*-retinoids from the incubation of [11,12-<sup>3</sup>H]9-*cis*-retinal or [11,12-<sup>3</sup>H]13-*cis*-retinol with frog retina/pigment epithelium washed and unwashed 600 × *g* supernatant at room temperature for 3 h

Form of [ <sup>3</sup> H]retinoid supplied	Retinol		Retinal		Retinyl palmitate		11- <i>cis</i> -Retinoid formed
	11- <i>cis</i>	Recovery	11- <i>cis</i>	Recovery	11- <i>cis</i>	Recovery	
	%		%		%		%
9- <i>cis</i> -Retinal (without membranes, <i>n</i> = 2) <sup>a</sup>			2.3 ± 0.6	>99			2.3
9- <i>cis</i> -Retinal (with unwashed membranes, <i>n</i> = 3) <sup>b</sup>	3.5 ± 0.7	10.4 ± 7.1	1.8 ± 0.1	62.8 ± 0.6	4.9 ± 2.0	26.8 ± 0.7	2.8
13- <i>cis</i> -Retinol (with unwashed membranes, <i>n</i> = 2) <sup>c</sup>	21.6 ± 0.6	27.7 ± 0.7	45.0 ± 1.7	12.1 ± 0.7	7.1 ± 2.1	60.2 ± 0.1	15.7
13- <i>cis</i> -Retinol (with washed membranes, <i>n</i> = 2) <sup>c</sup>	5.0 ± 0.5	11.8 ± 0.6	2.2 ± 0.1	24.4 ± 2.9 <sup>d</sup>	7.1 ± 1.3	63.8 ± 2.3	5.6
all- <i>trans</i> -Retinol (with washed membranes, <i>n</i> = 1) <sup>e</sup>	40.9	11.7	23.0	11.4	24.6	76.9	26.3

<sup>a</sup> All values are mean ± S.D.

<sup>b</sup> The incubation mixture included 300 μl of frog retina/pigment epithelium 600 × *g* supernatant, 0.2 μCi of [11,12-<sup>3</sup>H]9-*cis*-retinal, and 15 μl of 10% BSA.

<sup>c</sup> The incubation mixture included 300 μl of washed or unwashed 600 × *g* supernatant, 0.3 μCi of [11,12-<sup>3</sup>H]13-*cis*-retinol, and 15 μl of 10% BSA. 13-*cis*-Retinol is rather configurationally labile. In a control incubation without eye tissue, after 3 h at room temperature, 15% of the 13-*cis*-retinol was isomerized to all-*trans*-retinol.

<sup>d</sup> 86% of the retinal pool was in all-*trans*-retinal form. This indicates that 13-*cis*-retinol was isomerized to all-*trans*-retinal rapidly by the homogenate.

<sup>e</sup> The same amount of [11,12-<sup>3</sup>H]all-*trans*-retinol was used as substrate to provide a comparison.

not all, of the isomerization to 11-*cis*-retinoids can be accounted for by the "spontaneous" isomerization of the enzymatically formed 13-*cis*-retinal to all-*trans*-retinal, followed by the reduction of the all-*trans*-retinal to all-*trans*-retinol. Further evidence for this proposal comes from studies on the abilities of the membranes to isomerize added 13-*cis*-retinol to all-*trans*-retinol. Incubation of a 150,000 × *g* supernatant membrane preparation with 13-*cis*-retinol for 3 h led to the reisolation of 22% 13-*cis*-retinol and 73% all-*trans*-retinol. Dialysis of this membrane preparation markedly reduced the amount of isomerization so that 60% of 13-*cis*-retinol and 33% of all-*trans*-retinol were reisolated. Addition of 1 mM NAD/NADP to these latter membranes led to the depletion of 13-*cis*-retinol again so that only 14% of 13-*cis*-retinol was recovered compared to 84% of all-*trans*-retinol. In addition, it must be remembered that 13-*cis*-retinol is itself not configurationally stable and readily forms all-*trans*-retinol in solution (13).

**Attempted Solubilization of the Isomerase**—Even if it is likely that free all-*trans*-retinol is the substrate for isomerization, kinetic characterization of the retinoid isomerase system in a 600 × *g* supernatant is still highly problematic, because 80–90% of the all-*trans*-retinol substrate is removed by competing enzymatic activities very early in an isomerase assay. It is apparent that some sort of purification of the isomerase activity away from the ester synthetase and alcohol dehydrogenase activities would be a desirable next step. Previously published sedimentation experiments have indicated that the isomerization activity is membrane-bound (1). One approach to the purification of the isomerase would be to release it from the membrane by detergent solubilization, if it is an integral protein, or by treatment with high salt concentration or chelating agents, if it is membrane-associated. In either case, the possibility of denaturation must be carefully monitored.

In order to try to detect both solubilization and denaturation, the initial approach used was to add the solubilizing agent to a 600 × *g* supernatant and then to centrifuge the supernatant at 13,000 × *g*, a speed that partially sediments isomerizing activity from the supernatant phase (1). Thus, an

TABLE III  
Attempted detergent solubilization of isomerase activity

Experiment	Concentration	11- <i>cis</i> in retinol pool <sup>a</sup>
		%
No eye ( <i>n</i> = 5)		0.3
No additions ( <i>n</i> = 5)		21.0
+EDTA	1 mM	13.8
+KCl	500 mM	10.5
+CHAPS	0.1%	13.3
+CHAPS	1.5%	0.2
+Octyl glucoside	0.1%	11.1
+Octyl glucoside	1.5%	0.2
+Digitonin	0.1%	13.0
+Digitonin	1.0%	0.3
+Sodium deoxycholate	0.1%	1.4
+Sodium deoxycholate	1.0%	0.3
+Triton X-100	0.1%	3.0
+Triton X-100	1.0%	0.2
+Lubrol PX	0.1%	4.0
+Lubrol PX	1.0%	0.2

<sup>a</sup> 3 h incubation with [11,12-<sup>3</sup>H]all-*trans*-retinol.

increase in isomerizing activity in the 13,000 × *g* supernatant in the presence of a solubilizing agent would be an indication of successful solubilization, while a decrease or loss of activity would be an indication of denaturation. Table III shows that addition of EDTA or KCl to disrupt possible ionic interactions between membranes and membrane-associated proteins led to a partial loss of isomerizing activity. All six detergents shown in Table III completely inhibited the isomerase activity when used at concentrations of 1.0–1.5%, concentrations recommended for effective solubilization of membrane proteins (14). At 0.1% detergent concentration, all of the detergents inhibited activity, although not to such a complete extent.

**Percoll Gradient Purification of Pigment Epithelium Membranes**—Since the detergent solubilization studies indicate that the isomerase must remain in its native membrane to retain activity, an alternative purification method that works on native membranes, differential centrifugation, was tried. Percoll, a colloidal silica coated with polyvinylpyrrolidone,



was selected as the density gradient medium because it forms isoosmotic self-generated density gradients. Only rarely has it ever been found to interfere with enzyme assays (15). The material loaded onto the gradient was from membranes of frog pigment epithelium that had been pelleted at  $100,000 \times g$  in 0.25 M sucrose for 1 h. Gradients generated from 50, 30, 20, and 10% Percoll were tried, and 20% Percoll was determined to provide the optimal separation of isomerizing activity from the main peak of protein. All successful gradients utilized sonicated membrane preparations; when Teflon pestle homogenization was used in place of sonication there was inadequate activity to assay.

A 20% Percoll gradient of sonicated frog pigment epithelium membranes is shown in Fig. 3. Isomerase activity spreads throughout most of the gradient, but it can be seen that the maximum percent conversion of the retinol pool to the 11-*cis* configuration appears to be shifted to somewhat lower densities relative to the general protein on the gradient. In the bottom half of the gradient there is an enormous amount of ester synthetase activity, as measured by the ratio of extracted retinyl esters to retinol after incubation with [ $^3$ H]all-*trans*-retinol. At fraction 4, the peak of both the isomerase and the ester synthetase activities, more than 95% of the retinol has been converted to other retinoids. Although at fraction 4 there has been approximately 5-fold purification of isomerase activity with respect to protein, this fraction is functionally even more contaminated by ester synthetase activity than the starting material. It is of interest that at lower densities of the gradient there is residual isomerase activity that is relatively free of the contaminating ester synthetase activity. This suggests that when sonicating the tissue, the lighter membranes, possibly very small vesicles, may be relatively enriched in isomerizing activity. It thus may be possible to achieve a partial purification of the retinol isomerase activity from contaminating enzymatic activities by trying to isolate these light membranes.

**Isomerase Activity in a  $100,000 \times g$  Supernatant of Frog Retina/Pigment Epithelium**—The observation that the light membranes are enriched in isomerase activity relative to contaminating enzymatic activities was exploited as a possible functional purification of the isomerase system that would permit further enzymatic characterization. To prepare light

membranes, the frog retina/pigment epithelium membrane preparation was thoroughly sonicated. Centrifuging this preparation at  $100,000 \times g$  for 1 h in a preparative ultracentrifuge (equivalent to a  $150,000 \times g$  centrifugation for 10 min in a Beckman Airfuge) leaves approximately 5% of the isomerase activity in the supernatant (1). The recovery of retinol at the end of an isomerization assay is always greater than 50% and is often much higher (Table IV). The extent of esterification of the added all-*trans*-retinol is 5–10%, as opposed to between 80 and 90% with the  $600 \times g$  membrane preparation (1). The  $100,000 \times g$  supernatant fraction retains most of its isomerizing activity if stored for a week at  $4^\circ\text{C}$ , and it can be frozen if necessary. The specific formation of 11-*cis*-retinol occurs in a time-dependent fashion with these membranes and is linear for at least 2 h.

The assays of the  $100,000 \times g$  supernatant were optimized by determining the minimum amount of extract required for maximum activity. It was determined that isomerizing activity levels off above  $100 \mu\text{l}$  of  $100,000 \times g$  extract. Based on this determination and the previously described time course determinations, the standard conditions for all of the following experiments were chosen to be  $100 \mu\text{l}$  of extract assayed for 2 h.

**Identification of the Retinoid Substrate for Isomerase Activity in a  $100,000 \times g$  Supernatant**—The three possible free substrates for 11-*cis*-retinoid production, all-*trans*-retinol, all-*trans*-retinal, and all-*trans*-retinyl palmitate, were tested with the  $100,000 \times g$  supernatant. Table IV shows results from assays with [ $^3$ H]all-*trans*-retinol, [ $^3$ H]all-*trans*-retinal, and [ $^3$ H]all-*trans*-retinyl palmitate in the presence and absence of a  $100,000 \times g$  supernatant from frog retina/pigment epithelium. The  $\text{NH}_2\text{OH}/\text{CH}_2\text{Cl}_2$  total retinoid extraction method was used, and HPLC fractions were collected over time. The frequency of fraction collection was adequate to distinguish between the main types of retinoids, but not their isomeric compositions. When sufficient activity was present in a retinoid peak, the sample was reinjected in a peak detection mode to determine isomeric composition. These data demonstrate that all-*trans*-retinal and all-*trans*-retinyl palmitate are virtually inert in this preparation. Only all-*trans*-retinol is processed to 11-*cis*-retinoids, and 11-*cis*-retinol is the major product. These experiments clearly show that added all-*trans*-retinol is the preferred substrate for the retinoid isomerase activity in the  $100,000 \times g$  supernatants from frog retina/pigment epithelium.

**Kinetic Characterization and pH Optimum of Retinoid Isomerase Activity in a  $100,000 \times g$  Supernatant**—The enzyme

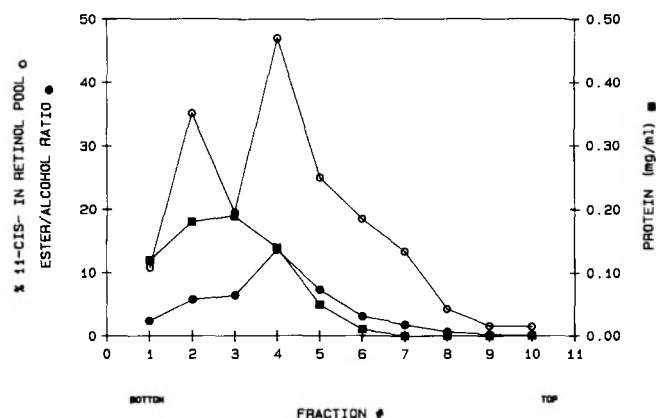


FIG. 3. 20% Percoll gradient of membranes from frog pigment epithelium membranes. A 20% Percoll gradient was performed on membranes from frog pigment epithelium as described under "Material and Methods." Gradient fractions were assayed for protein concentration and then incubated in the dark with [ $^3$ H]all-*trans*-retinol for 3 h under standard conditions. Retinoid extraction was by the  $\text{NH}_2\text{OH}/\text{CH}_2\text{Cl}_2$  method. The main band of protein sedimented at a density between 1.037 and 1.049 mg/ml, as measured by density marker beads.

TABLE IV  
Identification of the retinoid substrate for isomerase activity in a  $100,000 \times g$  supernatant

Form of [ $^3$ H]retinoid	Retinyl palmitate		Retinal		Retinol	
	11- <i>cis</i>	Recovery <sup>a</sup>	11- <i>cis</i>	Recovery <sup>a</sup>	11- <i>cis</i>	Recovery <sup>a</sup>
	%		%		%	
Retinol (control)		<1		<1	0.3	>99
Retinol ( $100,000 \times g$ supernatant)	3.1	8	5.4	32	3.4	60
Retinal (control)		<1	0.0	97		3
Retinal ( $100,000 \times g$ supernatant)		<1	0.0	93		7
Retinyl palmitate (control)	1.6	>99		<1		<1
Retinyl palmitate ( $100,000 \times g$ supernatant)	1.6	97		<1		3

<sup>a</sup> % total isomeric recovery of the particular retinoid relative to the total amount of retinoids recovered.

kinetic constants for the retinoid isomerase system were then determined by adding various concentrations of unlabeled all-*trans*-retinol to a standard assay mixture. The data, plotted in the graph of Fig. 4A demonstrate that the isomerizing system exhibits substrate saturation kinetics. The data, when replotted in Fig. 4B according to the method of Hanes ( $S/V$  versus  $S$ ) as recommended by Cornish-Bowden (16), yielded values for the apparent  $K_M$  of  $0.8 \mu\text{M}$  and a  $V_{\text{max}}$  of 5 pmol/h/mg of protein at a physiological pH of 7.2, when retinol was the substrate.

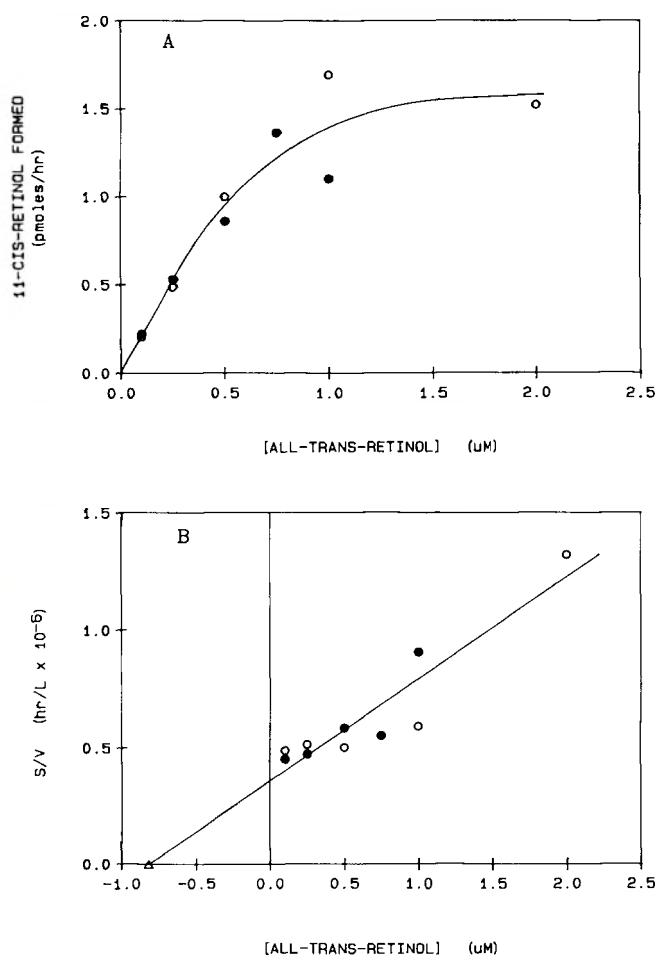
The retinoid isomerase system is active between pH 7.0 and 9.0, with an optimum at pH 8.0. In the pH optimum experiments a phosphate/citrate/borate buffer known to provide excellent buffering capacity from pH 2–12 (17) was used instead of the usual pH 7.2 50 mM phosphate buffer.

**Studies on the Energy Source for 11-*cis*-Retinoid Formation in  $100,000 \times g$  Supernatants**—In the experiments described above, no exogenous energy-source cofactors were ever required in order to observe isomerizing activity. Since the  $100,000 \times g$  supernatants had been dialyzed, this would imply that soluble, low molecular weight energy sources such as

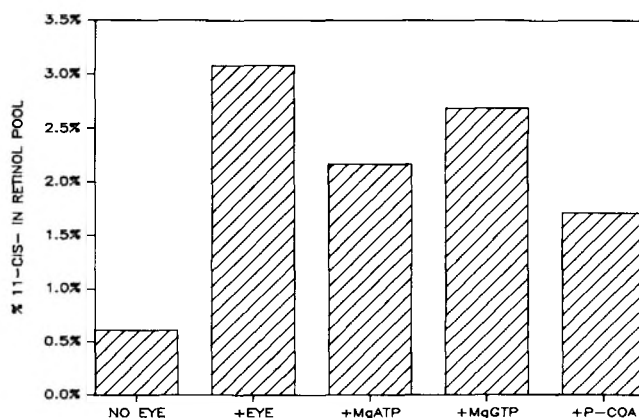
ATP, GTP, or palmitoyl-CoA would be unlikely to be cofactors. Nevertheless, it is possible that small amounts of such cofactors might be tightly bound and resistant to complete removal by dialysis. When MgATP, MgGTP, or palmitoyl-CoA was added to a  $100,000 \times g$  supernatant, there was no enhancement of isomerizing activity (Fig. 5). In other experiments on various preparations of retina and pigment epithelium, conditions designed to disrupt energy metabolism, such as  $\text{NaN}_3$ , 2,4-dinitrophenol, or a nitrogen atmosphere, never inhibited 11-*cis*-retinol production.

**11-*cis*-Retinol Production in  $100,000 \times g$  Supernatants Requires Intact Protein and Membranes**—As described above and previously (1), the retinoid isomerase has the characteristics of a membrane-bound enzyme. Several experiments on  $100,000 \times g$  supernatants also support this idea. Further Beckman Airfuge centrifugation of a  $100,000 \times g$  supernatant at  $150,000 \times g$  for 10 or 60 min caused a progressive loss of isomerizing activity from the supernatant fraction, and isomerizing activity progressively appeared in the  $150,000 \times g$  pellet (data not shown). In Fig. 6 it is shown that addition of proteinase K, addition of phospholipase C, or boiling for 5 min completely destroyed 11-*cis*-retinol forming activity. Proteinase K's inhibition of isomerization could be partially prevented by the simultaneous addition of the protease inhibitor PMSF. All of the experiments reported here are in accord with the idea that the retinoid isomerase involves a membrane-bound enzyme.

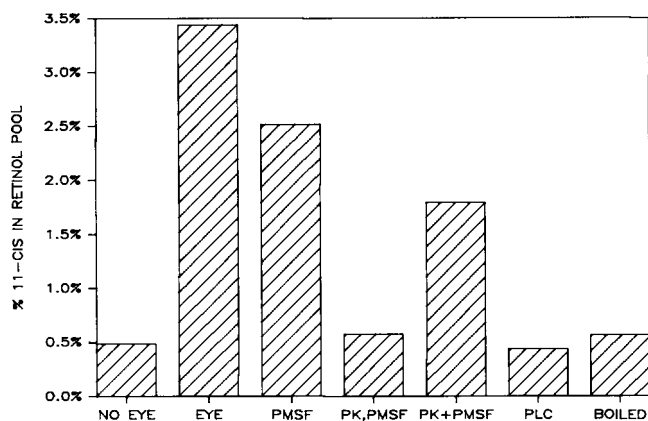
**Ethanol Can Inhibit 11-*cis*-Retinol Formation in a  $100,000 \times g$  Supernatant**—While trying to optimize the conditions for isomerization of all-*trans*-retinoids to 11-*cis*-retinoids, it was observed that it was very important to keep the ethanol in the assay mixture as low as possible. The maximum amount of ethanol, the solvent in which the  $[^3\text{H}]$ all-*trans*-retinol was stored, never exceeded 0.5% in any of the assays described here. Fig. 7 shows that at 1.0% ethanol, 11-*cis*-retinol production is inhibited by about 50%, and at 2 or 4% ethanol, isomerase activity is virtually undetectable. It should be noted that previous studies in which it was claimed that homogenates of pigment epithelium cannot isomerize added all-*trans*-retinoids to 11-*cis*-retinoids always used 2–4% ethanol in the assays (18, 19).



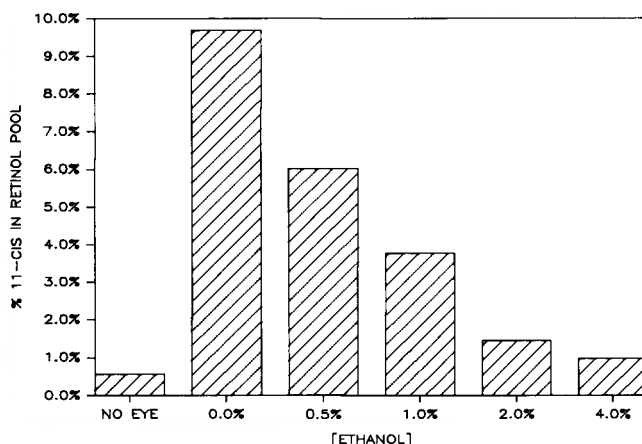
**FIG. 4. Kinetic parameters of retinol isomerization by  $100,000 \times g$  supernatants.** 100- $\mu\text{l}$  portions of a  $100,000 \times g$  supernatant were incubated with 100  $\mu\text{l}$  of phosphate buffer, 10  $\mu\text{l}$  of 10% BSA, 1  $\mu\text{Ci}$  of  $[11,12\text{-}^3\text{H}]$ all-*trans*-retinol in 1  $\mu\text{l}$  of ethanol, and sufficient unlabeled retinol to achieve the final concentrations indicated. Two independently prepared  $100,000 \times g$  supernatants were used.  $\circ$ , 3.33 mg of protein/ml;  $\bullet$ , 5.45 mg of protein/ml. Incubation in the dark was for 2 h, and retinoid extraction with hexane was used. A shows the amount of 11-*cis*-retinol formed as a function of all-*trans*-retinol concentration. B is a Hanes plot ( $S/V$  versus  $S$ ) (16) to determine the apparent enzyme kinetic parameters.



**FIG. 5. Energy source study.** 100- $\mu\text{l}$  portions of a  $100,000 \times g$  supernatant (3.96 mg of protein/ml) were mixed with 80  $\mu\text{l}$  of phosphate buffer. NO EYE and +EYE each received 20  $\mu\text{l}$  of phosphate buffer. +MgATP received 20  $\mu\text{l}$  of 13 mM ATP in 50 mM  $\text{MgSO}_4$ , and +MgGTP received 20  $\mu\text{l}$  of 13 mM GTP in 50 mM  $\text{MgSO}_4$ . +P-CoA received 20  $\mu\text{l}$  of 1 mM palmitoyl-CoA. 10  $\mu\text{l}$  of 10% BSA and 1  $\mu\text{Ci}$  of  $[11,12\text{-}^3\text{H}]$ all-*trans*-retinol supplied in 1  $\mu\text{l}$  of ethanol were added to each tube. Hexane extraction and HPLC analysis, after 2 h of dark incubation, were by standard methods.



**FIG. 6. Enzymatic inhibition of 11-cis-retinol formation.** 100- $\mu$ l portions of a 100,000  $\times$  g supernatant were incubated at room temperature for 1 h as follows: *NO EYE* and *EYE* each received 10  $\mu$ l of phosphate buffer and 10  $\mu$ l of 2% ethanol at the start of the incubation; *PMSF* received 10  $\mu$ l of phosphate buffer and 10  $\mu$ l of 25 mM PMSF in 2% ethanol at the beginning of the incubation; *PK, PMSF* received 10  $\mu$ l of 1 mg/ml proteinase K (Sigma) at the beginning of the incubation, and then received 10  $\mu$ l of 25 mM PMSF in 2% ethanol at the end of the 1-h incubation; *PK+PMSF* received 10  $\mu$ l of 1 mg/ml proteinase K and 10  $\mu$ l of 25 mM PMSF in 2% ethanol at the start of the incubation; *PLC* received 2 units of phospholipase C (Sigma, from *Claustidium perfringens*) in 20  $\mu$ l of phosphate buffer at the start of the 1-h incubation; *BOILED* received 20  $\mu$ l of phosphate buffer and was then boiled for 5 min. At the end of the 1-h incubation, 80  $\mu$ l of phosphate buffer was added to each tube, as were 10  $\mu$ l of 10% BSA and 1  $\mu$ Ci of [11,12- $^3$ H]all-*trans*-retinol in 1  $\mu$ l of ethanol. Standard hexane extractions and HPLC analyses were done after 2 h of dark incubation. All values are the average of at least two independent experiments with different 100,000  $\times$  g supernatant preparations.



**FIG. 7. Effect of ethanol on isomerization.** 1  $\mu$ Ci of [11,12- $^3$ H]all-*trans*-retinol in 1  $\mu$ l of ethanol was added to each assay tube and then dried down under a stream of nitrogen. 100  $\mu$ l of 100,000  $\times$  g supernatant (5.40 mg of protein/ml) was added, followed by 100  $\mu$ l of phosphate buffer containing enough ethanol to yield the final concentrations shown. 10  $\mu$ l of 10% BSA was also added to each tube. After 2 h of dark incubation, standard hexane extraction and HPLC analysis were performed.

#### DISCUSSION

Previously, we have shown that membranes from amphibian retina/pigment epithelium can partially transform added all-*trans*-retinol to a mixture of 11-*cis*-retinol(palmitate) and 11-*cis*-retinal (1). The isomerase activity proved to be heat-unstable and located largely, if not exclusively, in the pigment

epithelium of the amphibian (1). Using a washed 600  $\times$  g membrane fraction to remove soluble cofactors, we found that added all-*trans*-retinol was by far the preferred substrate (1). The current studies began by extending these previously published results.

To begin with, a full time course for the processing of added [ $^3$ H]all-*trans*-retinol by the membranes was performed (Fig. 1, A and B). Here it can be seen that the membranes rapidly esterify approximately 80% of the added substrate, forming all-*trans*-retinyl palmitate. The full range of 11-*cis*-retinoids were produced more slowly and in a time-dependent fashion. Given the metabolic complexity of the membranes with respect to the metabolism of retinol, it is not immediately apparent which retinoid form is the actual isomerase substrate.

To further elucidate this point, a double-labeling method, already employed in *in vivo* studies to reveal the oxidation state of the isomerase substrate (10), was employed here. The fate of the 15- $^3$ H of nonstereospecifically labeled [15- $^3$ H]- and [15- $^{14}$ C]all-*trans*-retinol was followed during its conversion to 11-*cis*-retinoids (Fig. 2, A-C). In two different preparations of the amphibian membranes and in membranes from bovine pigment epithelium (previous results from this laboratory have already established that there is isomerase activity in the bovine pigment epithelium membranes, but not in the retina (8)), no loss of tritium in the 11-*cis*-retinoids was observed. These experiments are in complete accord with our *in vivo* results on the rat (10) and establish beyond a doubt that free all-*trans*-retinal cannot be the isomerase substrate in three different species, rat, frog, and cow.

The metabolic complexity of these membranes with respect to retinol processing is apparent from the results shown in Fig. 1. These membranes, however, will not convert added all-*trans*-retinyl palmitate into 11-*cis*-retinoids (1). On the surface, this result might be interpreted to mean that esters are not substrates for the isomerase and that the free all-*trans*-retinol must be the substrate. However, the extreme hydrophobicity of the palmitate esters makes them virtually inert to intermembranous exchange (12). By contrast, the retinols and retinals undergo virtually instantaneous intermembranous exchange (12). It is consequently in the realm of possibility that the reason why the added all-*trans*-retinyl palmitate is not processed by the membranes is that it cannot get to where the isomerase is located. This possibility is made more feasible by the recent observation that pigment epithelium membranes show esterase activity against all-*trans*-retinyl palmitate only in the presence of a detergent (20). In the present situation detergents cannot be used because of the apparent sensitivity of the isomerase to them (Table III). With this in mind, we explored the possibility that all-*trans*-retinol esters less hydrophobic than the palmitate ester might be isomerized. We found that the non-naturally occurring acetate, butyrate, and hexanoate esters of all-*trans*-retinol were all hydrolyzed, though at different rates, by esterase activity in the membranes (Table I). However, no evidence was found for the *direct* isomerization of an all-*trans*-retinyl ester to an 11-*cis*-retinyl ester. From all of these experiments it is concluded that the isomerase cannot directly isomerize an all-*trans*-retinyl ester into its 11-*cis* congener.

The results described above are in complete accord with the hypothesis that free all-*trans*-retinol is importantly involved as a substrate in the isomerization process. Whether the free retinol is further converted prior to isomerization into another metabolite which is at the same oxidation state as retinol is at this point not known. Nevertheless, the substrate specificity for the isomerase with respect to the isomeric



retinols was further explored. As is clear from Table II, 9-*cis*-retinol(al) is not an isomerization substrate. The situation with 13-*cis*-retinol is more complex (Table II). Here it was found that unwashed membranes could produce 11-*cis*-retinoids from this isomer nearly as well as from all-*trans*-retinol (1). However, washing the membranes, which removes nicotinamide-based redox cofactors, strongly suppressed the amount of isomerization of the added 13-*cis*-retinol (Table II). It is noteworthy that this washing treatment had previously been shown not to affect the extent of isomerization from all-*trans*-retinol (1). Both 13-*cis*-retinol and 13-*cis*-retinal are "spontaneously" isomerized to their all-*trans* counterparts, the latter much more rapidly than the former (13). This means that all-*trans*-retinol can be formed here, both by the direct isomerization of 13-*cis*-retinol and by the isomerization, followed by reduction, of enzymatically produced 13-*cis*-retinal. However, the possibility that 13-*cis*-retinol might be a weak substrate for the isomerase cannot be ruled out.

These results are of interest in light of some of our previous *in vivo* studies in the rat, where we found that 13-*cis*-retinol was converted into 11-*cis*-retinoids nearly as well as all-*trans*-retinol was (9). The *in vitro* results described here both corroborate and explain these earlier experiments. The physiological significance of the 13-*cis* isomerization pathway is not completely clear at this time. A possibility is that it is a salvage pathway which recovers any spontaneously formed 13-*cis* isomers in the eye.

The work described above on the isomerase made use of a crude 600 × *g* supernatant fraction (1). The rapid removal of 80–90% of the [<sup>3</sup>H]all-*trans*-retinol substrate at the beginning of an isomerase assay by the crude membranes greatly complicated any further characterization of the retinol isomerase activity, making it clear that some sort of purification should be tried. Attempts at solubilizing the activity in detergents were unsuccessful, because loss of isomerase activity always occurred (Table II), precluding the use of chromatographic separations at this time.

Light membranes found in a 100,000 × *g* supernatant of frog retina/pigment epithelium were chosen as the best partially purified tissue preparation for further characterization because they appeared to be significantly enriched in isomerase activity relative to ester synthetase activities and retinol dehydrogenase. Table IV shows that when [<sup>3</sup>H]all-*trans*-retinol was added to this preparation, production of 11-*cis* retinoids occurred, and it was clear that, although there was residual ester synthetase and retinol dehydrogenase activity, they were not present in nearly the same amounts seen in 600 × *g* supernatants (1). [<sup>3</sup>H]all-*trans*-Retinyl palmitate was virtually inert in the experiments of Table IV. Very little hydrolysis was seen, because the known esterases that can hydrolyze all-*trans*-retinyl palmitate at neutral pH are active only in the presence of detergents (20–22). [<sup>3</sup>H]all-*trans*-Retinal was likewise inert in the experiment of Table IV. Apparently, this dialyzed 100,000 × *g* supernatant, unlike a 600 × *g* supernatant (1), has virtually no remaining retinal reductase activity. These experiments clearly show that all-*trans*-retinol is the preferred substrate in this 100,000 × *g* supernatant, a result consistent with previous studies (1).

Kinetic characterization of the retinol isomerase activity with a [<sup>3</sup>H]all-*trans*-retinol substrate at a physiological pH of 7.2 gave values for the apparent  $K_M$  and  $V_{max}$  of 0.8 μM and 5 pmol/h/mg of protein, respectively (Fig. 4, A and B). The apparent  $K_M$  is in the range commonly found for other retinoid-metabolizing enzymes of the pigment epithelium (21, 23). The apparent  $V_{max}$  is quite low, but it must be remembered that approximately 95% of the isomerase activity has

been discarded in making this enzyme preparation from a 600 × *g* supernatant. Additionally, any rate-limiting cofactors are unknown. All of these kinetic values must be interpreted with caution when dealing with insoluble substrates and membrane-bound enzymes, because effective concentrations within the lipid phase are certainly much higher. It was also found that the isomerase system is active from pH 7–9, with an optimum at pH 8.0. Based on the  $V_{max}$  of 5 pmol/h/mg of protein, the fact that one eye yields about 1 mg of 100,000 × *g* supernatant, and the estimate of 95% loss of isomerizing activity in preparing 100,000 × *g* supernatant from a 600 × *g* supernatant, it can be calculated that a 600 × *g* supernatant would have an expected  $V_{max}$  of 100 pmol/h/eye. This value is a lower limit value because substantial (about one-half) isomerase activity remains in the 600 × *g* pellet, and activity is lost during homogenization. In living *Rana pipiens* the rate of 11-*cis*-retinoid synthesis during dark adaptation is 1–2 nmol/h/eye (24).

The isomerization process described here is unique in many respects. One interesting aspect of the isomerase, which makes it unlike any previously described isomerase, is the fact that it catalyzes a thermodynamically uphill process. 11-*cis*-Retinoids are approximately 4 kcal/mol less stable than their all-*trans* counterparts (25). No energy source for this endergonic isomerization process has yet been identified (Fig. 5). So far, only the requirement for intact protein and membranes has been demonstrated (Fig. 6). The ability to sediment the isomerase activity argues against the involvement of any of the known retinoid binding proteins, all of which are soluble proteins (26, 27). If a small water-soluble cofactor is an energy source it must be quite resistant to removal by dialysis or sedimentation. Perhaps the energy source is a lipid-soluble cofactor or a previously unknown membrane-bound retinoid binding protein, or it could be coupled in some manner to ester hydrolysis after *in situ* esterification of the added [<sup>3</sup>H] all-*trans*-retinol. A similar difficulty in identifying an energy source has been encountered in the study of the retinyl ester synthetase of the eye (23).

All experiments thus far have been unable to remove completely all of the ester synthetase activity from the retinol isomerase activity, so it is possible that isomerization and retinyl ester synthesis may be linked, perhaps as a multi-enzyme complex. The free energy of retinyl ester hydrolysis could, of course, provide the needed energy to drive the endothermic isomerization because it would be expected to yield more than 4 kcal/mol (28). In bovine pigment epithelium membranes, we have noted an intriguing relationship between retinyl ester synthesis and isomerization because a diverse group of reagents, including ethanol, hydroxylamine, and *p*-hydroxymercuribenzoate, inhibit retinyl ester synthesis and isomerization in a parallel manner (8).

The experiments showing that ethanol inhibits 11-*cis*-retinol formation in eye tissue homogenates (Fig. 7) are quite important because they explain why Bridges and co-workers (18, 19), using similar homogenates of pigment epithelial cells, had previously not detected any isomerase activity. In these published reports, these investigators always used 2–4% ethanol in their incubations, an amount sufficient to suppress isomerizing activity almost completely. In the experiments reported here, and in our previously published experiments, the ethanol concentration was never above 0.5%, and bovine serum albumin was added as a non-denaturing retinoid carrier (1). Using these methods Bridges and Alvarez (29) have recently confirmed our observations on the ability of pigment epithelium membranes to biosynthesize 11-*cis*-retinoids from added all-*trans*-retinol.

Further purification of the retinoid isomerase system will clarify its mechanism of action, its energy source, and its other requirements. Nevertheless, even in its current state, it is clear that the isomerase is biologically relevant, with an almost certainly important role to play in the physiology and pathophysiology of vision in the vertebrate eye.

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